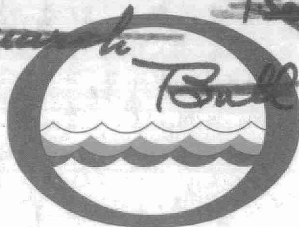


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THE ISOLATION OF VIRUS

(BACTERIOPHAGE)

FROM WATER AND WASTEWATER

BY THE

MILLIPORE FILTER METHOD

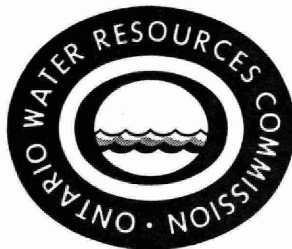
THE ONTARIO WATER RESOURCES COMMISSION

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THE ISOLATION OF VIRUS (BACTERIOPHAGE)
FROM WATER AND WASTEWATER BY THE
MILLIPORE FILTER METHOD

By

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INTRODUCTION

The increased re-use of water to meet the expanding demand for domestic supplies has resulted in concern regarding the presence of pathogenic viruses in potable water (1, 2). A satisfactory virus-isolation technique which is economical enough for routine application and which meets universal approval with respect to reproducibility, has not yet been found.

A simple, inexpensive method which has received recent attention (3), involves the use of standard Millipore filters*. The results of only a few experiments using raw waters were reported however, and none were apparently attempted using finished (treated) water; in addition, the volumes of water used did not exceed 500 ml. Since the estimated number of viruses in surface waters, even of a polluted nature, would be lower than the levels which were tested (25-35 per 500 ml minimum), there was a necessity to evaluate the method for lower virus numbers in larger volumes of water.

*Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.

This report gives the results of this evaluation, and also those obtained when the method was applied to the isolation of viruses from waste waters.

MATERIALS AND METHODS

Virus

The virus utilised in these experiments was a bacteriophage of E coli B, prepared and purified according to the method of Adams (4). Virus stocks were stored at +4°C until ready for use, when they were diluted to the required concentration.

Virus Assay

The virus was enumerated using a modified most probable number (MPN) method (5), first described by Kott (6), which is particularly applicable where low numbers of virus are present. Briefly, five samples of 10 ml, five samples of 1 ml and five samples of 0.1 ml of virus suspension are inoculated into broth tubes, which are then seeded with E coli B, the host organism. After growth overnight at 35°C, the tubes containing virus are detected by plating a loopful from each on to an indicator plate freshly seeded with the host organism; the presence of virus is indicated by a clearing in the growth of the host organism on the plate. Virus numbers per 100 ml of original suspension can then be computed by referring the combination of virus-positive tubes to

the probability tables for coliforms. The number of virus per ml of appropriately diluted virus stock was determined by adding 1 ml to 100 ml of distilled water and assaying by the MPN technique. Virus recoveries were estimated by adding the whole 10 ml of eluate obtained after filtration and elution (see "Filtration Technique", page 6), to 90 ml of distilled water, and assaying by the MPN technique.

Media

- a) Virus dilutions were carried out in dilution water (7).
- b) 3% beef extract (Difco) was used to elute the virus from the membrane filter, made up and sterilized according to directions.
- c) Phage assay broth (PAB) was Difco lauryl tryptose broth made up according to directions; CaCl_2 was not added in this series of experiments. The host organism, E coli B, was grown in nutrient broth (Difco) for five hours at 35°C prior to seeding into the PAB tubes.
- d) Indicator plates were phage assay agar (PAA) consisting of 8 gm Difco nutrient broth, 5 gm NaCl and

15 gm Difco agar per litre. They were seeded with a drop of a heavy suspension prepared from an overnight growth of E coli B on a nutrient agar (Difco) slant, made up in dilution water.

Contamination of Water Samples

Contaminated water samples were prepared by adding the appropriate dilution of virus to the required volume of the water; volumes of various waters and waste waters up to 20 litres were tested. Waste waters, since they were likely to contain E coli B bacteriophage, were autoclaved before testing; all other waters were free of the virus before contamination.

To some water samples, 200 mg/litre $\text{Ca}(\text{NO}_3)_2$ were added, since it was reported (3) that Ca^{++} ions enhance virus adsorption to the membranes, and all samples were adjusted to approximate neutrality where necessary. Water was obtained from a drilled domestic well (140 feet deep) both before and after passage through a standard water softener. Surface waters from a rural pond, a rural creek, and from a river flowing through an urban area were also tested. To determine

the applicability of the method in the examination of finished waters, Toronto tap water was tested directly and after dechlorination; dechlorination was accomplished by bubbling air through the tap water, until no measurable chlorine residual remained.

The chemical characteristics of the waters tested are shown in Appendix 1.

Several test isolations were completed using autoclaved sewage effluents as the suspending medium for the test virus; much smaller volumes of such effluents were used, since rapid clogging of even the pre-filter occurred, as a result of suspended debris.

Filters

Membrane filters HA type, 0.45/ μ pore size, 47 mm diameter were used. With the more turbid waters, fibreglass pre-filters (Reeve Angel, Grade 934AA) were employed; these were left in position when elution was carried out.

Filtration Technique

Contaminated samples were filtered through a standard Millipore filter unit under a vacuum of

approximately 18 inches of mercury. Upon completion of filtration, a sterile tube was inserted into the vacuum flask with forceps and 10 ml of 3% beef extract was drawn through the filter to yield the virus eluate, which was assayed after aseptic removal of the tube from the apparatus.

RESULTS

The results of the recovery of virus from various volumes of distilled water with added Ca^{++} ions (200 mg/litre $\text{Ca}(\text{NO}_3)_2$) are shown in Table 1. This amount of calcium was used, rather than the 200 ppm Ca^{++} used by the previous workers (3), since it corresponds more closely to that found in tap water (see waters 6 and 7, Appendix 1). Recovery efficiency varied fairly widely for no apparent reason (Table 1). However, the method did allow detection of virus in 20 litres of water to which only 5 virus particles had been added, and low virus numbers could be consistently detected from 2, 5 or 10 litre volumes. On a practical basis, since even a single virus particle may be capable of initiating human infection, the ability of a method to detect low numbers of virus in large volumes of water is probably more important than an ability to recover quantitatively larger numbers.

Since considerable success in virus isolation was obtained using distilled water with added Ca^{++} ions, similar experiments were carried out using various other waters.

The recoveries of virus from finished water i.e. Toronto tap water, are shown in Table 2. The approximate chlorine residual measured by the OTA (orthotolidine-arsenite) method was 0.1 ppm chlorine; the pH was adjusted to neutrality by the addition of dilute hydrochloric acid, and no pre-filter was used. As can be seen, over 100 virus particles had to be present before the virus could be detected in the eluate, even in a volume of water as small as 1 litre. Recoveries from dechlorinated tap water were better, but somewhat erratic; as few as 5 particles per litre were wholly recovered, whereas 22 viruses per litre were not detected in another sample.

Results of experiments conducted on domestic-well water are shown in Table 3; no pre-filter was necessary to process this water. Again the recoveries do not appear to be as efficient as those obtained with distilled water. However, the probability of detecting 10 virus particles or more, from 1 litre of water appears to be good. Recovery was as efficient from the water after passage through the water softener; the unit reduced iron from 0.9 to 0.15 ppm, total organic carbon tenfold, and hardness from 302 to 114 ppm.

Table 4 records the results of isolation experiments using surface waters, all of which required the use of pre-filters. Recoveries were not quantitative, but virus was recovered from a 1 litre sample to which only 2 virus particles had been added.

It was found that the amount of a given sewage effluent that could be filtered within a reasonable length of time, varied with the source and quality. Since such effluents have been found to contain up to 70 virus particles per litre (8), a much smaller volume will suffice for testing, and 100 ml to 200 ml is probably sufficient in most cases and will filter rapidly without causing clogging. Good recoveries were obtained, the results being shown in Table 5.

DISCUSSION

The results obtained when virus was recovered from distilled water containing added Ca^{++} ions, indicate that the MF method can be an extremely sensitive technique for the detection of low virus numbers. However, when natural waters were used in isolation experiments, isolation efficiency was decreased. The presence of organic materials appears not to be the reason for this decreased efficiency, since dechlorinated tap water, creek water and well water after softening all contain low levels of total organic carbon (TOC), and their isolation efficiencies are apparently not different from pond or river water with high TOC contents. The non-recovery of the virus from the "finished" tap water samples is not due to its inactivation by the low chlorine residual; previous experiments in this laboratory have shown that the bacteriophage would remain unaffected by such a residual, at least for the length of time required for the filtration of the sample. There was no apparent decrease in recovery efficiency after passage of the well water through a softener.

The reason for the decreased efficiency of recovery of virus from natural waters compared to that from distilled water, is not known. It is possible that the presence of certain inorganic ions may prevent the adsorption of the virus to or its subsequent elution from the filter.

CONCLUSIONS

The MF method is the simplest technique thus far described for the isolation of viruses from water. Bacteriophages have often been employed as models for the behaviour of other types of virus, because of a similarity in physical properties. Thus, although this evaluation was performed using such a virus a comparable result would be expected from enteric viruses.

Excellent recoveries can be obtained with distilled water containing added Ca^{++} ions, even in volumes of 10 litres or more, but the efficiency of the process decreases when natural waters are used. The factors interfering with the isolation of virus by the process were not determined, but they are apparently unrelated to alkalinity, chlorides, iron or total organic carbon.

Recoveries are not as efficient as those obtained previously with soluble alginate filters (9), where larger numbers of virus were tested in smaller volumes (100 ml) of water. However, alginate filters are more expensive and suffer from the disadvantage of

clogging rapidly when large volumes of water are to be processed.

The method does not appear to be applicable to recoveries of virus from finished i.e. chlorinated waters, where recovery efficiencies were low and generally erratic. With other types of natural water, the recovery of virus in low numbers although more reliable, was not quantitative. However, the lack of quantitation may have been magnified by the use of a MPN method for estimating virus input and recovery levels.

Recovery of low numbers of virus from relatively small volumes of sewage was successful.

RECOMMENDATIONS

Although investigation of other methods of virus isolation are proceeding, the MF method would be recommended as the method of choice for any virus surveillance programme, at the present time. The MF technique makes possible the examination of large volumes of surface waters, for the presence of viruses.

The method is also applicable to the examination of sewage effluents.

TABLE 1
RECOVERY OF VIRUS (BACTERIOPHAGE) FROM CONTAMINATED
DISTILLED WATER WITH ADDED Ca^{++} (200 mg/l $\text{Ca}(\text{NO}_3)_2$) (7)

Volume of Water	Virus Input	95% Confidence Limits	Virus Recovery	95% Confidence Limits
1 litre	23	7 → 70	2	<0.5 → 7
1 litre	12	3 → 28	8	1 → 19
1 litre	12	3 → 28	2	<0.5 → 7
1 litre	2	<0.5 → 7	2	<0.5 → 7
1 litre	2	<0.5 → 7	2	<0.5 → 7
2 litres	23	7 → 70	17	5 → 46
2 litres	17	5 → 46	8	1 → 19
5 litres	23	7 → 70	13	3 → 31
5 litres	5	<0.5 → 13	5	<0.5 → 13
10 litres	94	28 → 220	13	3 → 31
10 litres	22	7 → 67	5	<0.5 → 13
20 litres	5	<0.5 → 13	7	1 → 17

TABLE 2

RECOVERY OF VIRUS (BACTERIOPHAGE) FROM CONTAMINATED

TAP WATER, CHLORINE RESIDUAL 0.1 ppm

	Volume of Water	Virus Input	95% Confidence Limits	Virus Recovery	95% Confidence Limits
TAP WATER	1 litre	43	15→114	0	-
	1 litre	49	17→130	0	-
	1 litre	79	25→170	0	-
	1 litre	542	180→1400	130	35→300
DECHLORINATED TAP WATER (6)	1 litre	49	17→130	33	11→93
	1 litre	240	68→750	8	1→19
	1 litre	23	7→70	2	<0.5→7
	1 litre	5	<0.5→13	5	<0.5→13
	1 litre	22	7→67	0	-

TABLE 3
RECOVERY OF VIRUS (BACTERIOPHAGE) FROM
CONTAMINATED WELL WATER

	Volume of Water	Virus Input	95% Confidence Limits	Virus Recovery	95% Confidence Limits
BEFORE SOFTENER (1)	1 litre	109	31→250	11	2→25
	1 litre	94	28→220	17	5→46
	1 litre	46	16→120	7	1→17
	1 litre	46	16→120	49	17→130
	1 litre	11	-	2	<0.5→7
	1 litre	5	<0.5→13	0	-
	1 litre	2	<0.5→7	0	-
	1 litre*	46	16→120	8	1→19
AFTER SOFTENER (2)	1 litre	109	31→250	4	<0.5→11
	1 litre	94	28→220	8	1→19
	1 litre	46	16→120	11	2→25
	1 litre	46	16→120	7	1→17
	1 litre	11	-	2	<0.5→7
	1 litre	5	<0.5→13	0	-
	1 litre	2	<0.5→7	2	<0.5→7
	1 litre	46	16→120	11	2→25

*not adjusted to pH 7.0, pH 7.8

TABLE 4
RECOVERY OF VIRUS (BACTERIOPHAGE) FROM
CONTAMINATED SURFACE WATERS

Type of Water	Volume of Water	Virus Input	95% Confidence Limits	Virus Recovery	95% Confidence Limits
RIVER (3)	1 litre	23	7→70	5	<0.5→13
	1 litre	79	25→190	8	1→19
	1 litre	94	28→220	13	3→31
POND (4)	1 litre	23	7→70	2	<0.5→7
	1 litre	46	16→120	7	1→17
	1 litre	2	<0.5→7	2	<0.5→7
	500 ml	79	25→190	8	1→19
	1 litre*	46	16→120	2	<0.5→7
CREEK (5)	1 litre	2	<0.5→7	0	-
	1 litre	46	16→120	13	3→31
	1 litre*	46	16→120	8	1→19

*not adjusted to pH 7.0

TABLE 5
RECOVERY OF VIRUS (BACTERIOPHAGE) FROM
CONTAMINATED SEWAGE EFFLUENTS

Type of Effluent	Volume of Effluent	Virus Input	95% Confidence Limits	Virus Recovery	95% Confidence Limits
PRECHLORINATION	500 ml	2	-	2	<0.5→7
	500 ml	10	-	2	<0.5→7
	500 ml*	10	-	0	-
	500 ml	2	-	5	<0.5→17
	100 ml	17	-	2	<0.5→7
	100 ml*	46	16→120	8	1→19
POST CHLORINATION	500 ml	2	-	5	<0.5→17
	500 ml	10	-	2	<0.5→7
	500 ml*	10	-	0	-
	500 ml	2	-	5	<0.5→17
	100 ml	17	-	2	<0.5→7

*not adjusted to pH 7.0, pH about 8.5

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APPENDIX 1
CHEMICAL CHARACTERISTICS OF WATERS TESTED

Type	ppm as CaCO ₃ Hardness	ppm as CaCO ₃ Alkalinity	ppm as Fe Iron	ppm as Cl	Initial pH	ppm Total Organic Carbon
1. Well Water - Before Softener	302	280	0.90	4	7.7	10.5
2. Well Water - After Softener	114	277	0.15	4	7.7	2.0
3. River Water	216	178	0.70	47	8.0	24.0
4. Pond Water	212	66	0.05	37	8.3	17.5
5. Creek Water	264	260	0.35	2	8.2	3.5
6. Dechlorinated Tap Water	148	94	0.05	31	8.4	4.5
7. Distilled Water + 200 mg/l Ca(NO ₃) ₂	108	7	-	1	7.8	1.0



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